Reduced GABAergic inhibition of kidney-related PVN neurons in streptozotocin-treated type 1 diabetic mouse

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Abstract

J Neurophysiol 110: 2192–2202, 2013. First published August 21, 2013; doi:10.1152/jn.00013.2013.—Activity of presympathetic neurons in the paraventricular nucleus (PVN) of the hypothalamus is known to play an important role in the regulation of sympathetic outflow. Sympathetic overactivity is associated with many pathophysiological conditions such as diabetes mellitus and hypertension; however, the underlying synaptic mechanisms are poorly understood. In this study, we examined the GABAergic inhibitory synaptic control of kidney-related presympathetic PVN neurons in the streptozotocin-treated type 1 diabetic mouse model, using patch-clamp slice electrophysiology in combination with retrograde labeling. Type 1 diabetes resulted in decreased frequency of miniature inhibitory postsynaptic currents (mIPSCs). Our data also demonstrated a reduction of mIPSC amplitude and mean inhibitory current without alteration of input resistance. Furthermore, our data revealed decreased tonic GABAergic inhibition of kidney-related PVN neurons in diabetic conditions, which was consistent with the observed increased excitability of the presympathetic PVN neurons. In summary, our data demonstrated decreased phasic and tonic inhibitory control of kidney-related presympathetic PVN neurons that suggest altered sympathetic circuitry in type 1 diabetes.

Prenoncultural Nervous System (PNS) Neurons in the paraventricular nucleus (PVN) of the hypothalamus play a significant role in the control of autonomic nervous system, thus influencing homeostatic functions (Chen and Toney 2003; Ciriello et al. 1984; Huang and Weiss 1999; LaGrange et al. 2003; Park et al. 2009; Swanson and Sawchenko 1980; Uyama et al. 2004; Yi et al. 2010). Presympathetic neurons in the PVN, through their projections to the brain stem and spinal cord, govern sympathetic outflow (Chen and Toney 2010; Kenney et al. 2003; Li and Pan 2007a; Pyner 2009).

Increased sympathetic activity is a well-known characteristic of many pathophysiological conditions including diabetes mellitus and hypertension, and impairment of presympathetic PVN neurons could contribute to the development and progression of elevated sympathetic outflow. For instance, the kidney is regulated by the renal sympathetic nerves, and any alteration in the renal sympathetic activity impacts the tone of renal vessels and thereby affects blood pressure (Mifflin 2001).

In the central nervous system, excitatory and inhibitory inputs regulate the activity of neurons. In vivo studies established that bilateral microinjection of GABA_A receptor agonists into the PVN decreased baseline renal sympathetic nerve activity, heart rate, and mean arterial pressure (Li and Pan 2007b; Zhong et al. 2008). In contrast, administration of GABA_A receptor antagonists into the PVN increased baseline renal sympathetic nerve activity and blood pressure (Kannan and Yamashita 1985; Li and Pan 2006; Zhong et al. 2008), indicating an existing persistent inhibition of presympathetic PVN neurons. PVN hyperactivity is known to increase sympathetic nerve activity in hypertensive rats (Cirillo et al. 1984; Herzig et al. 1991; Takeda et al. 1991), and removal of GABAergic inhibition results in augmented glutamatergic inputs within the PVN (Li and Pan 2007a, 2007b). These observations indicate the importance of GABAergic inhibition of presympathetic PVN neurons and suggest that decreased GABAergic inhibition could play a role in the elevation of sympathetic activity.

At the cellular level, excitatory and inhibitory synaptic currents could be divided into conventional phasic and persistent tonic currents (Nusser and Mody 2002; Okamoto et al. 2009). Phasic synaptic currents are outcome of neurotransmitter release at the synaptic cleft that activates ionotropic receptors, resulting in postsynaptic currents. On the other hand, neurotransmitter diffusing away from the synaptic cleft after being released can activate extrasynaptic ionotropic receptors. Continuous activation of extrasynaptic ionotropic receptors generates a persistent, tonic current. Although the role of tonic current is still largely speculative, this tonic current may play a role in synaptic integration by changing membrane input conductance and neuronal excitability (Nusser and Mody 2002; Nusser et al. 1995, 1998; Semyanov et al. 2003, 2004).

Diabetes mellitus, obesity, and metabolic syndrome are among the risk factors in the development of increased sympathetic activity that could contribute to the development and progression of hypertension. Interestingly, already in the early stages of the disease, autonomic dysfunction has been observed in diabetic patients (Spallone et al. 1994). Because the sympathetic outflow is increased in diabetic conditions and GABAergic inhibitory control of presympathetic PVN neurons plays an important role in the regulation of sympathetic outflow, we hypothesized that the inhibitory regulation of kidney-related presympathetic PVN neurons are reduced in streptozotocin-treated hyperglycemic mice.

In the present study, by using pseudorabies virus (PRV-152), we identified kidney-related presympathetic PVN neurons and determined the consequences of type 1 diabetes on the GABAergic inhibitory control. Our study demonstrated increased excitation and reduced inhibitory control of kidney-
related presympathetic PVN neurons in streptozotocin-treated hyperglycemic mice. This finding provides evidence that altered GABAergic regulation could contribute to the development of increased sympathetic outflow during diabetes.

MATERIALS AND METHODS

Animals. Male CD1 mice (7–8 wk old; Harlan Laboratories) were used in all experiments. Experiments were performed following the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by Tulane University’s Institutional Animal Care and Use Committee.

PV-152 injection. A retrogradely transported pseudorabies viral vector (PV-152, supplied by Dr. L. W. Enquist) that expresses enhanced green fluorescent protein (EGFP) was used to identify kidney-related neurons. Under anesthesia, the left kidney was exposed and ~4 μl of PV-152 were injected into the left renal parenchyma (2 injections at 2 sites). A drop of adhesive “liquid bandage” was used to seal each injection to prevent the leakage of the virus. The animals were maintained in a biosafety level 2 facility up to 88–98 h postinjection.

Streptozotocin injection. The mice were fasted overnight and then injected intraperitoneally with streptozotocin (STZ; 200 mg/kg) dissolved in 0.1 mol/l citrate buffer. Body weight and blood glucose level (OneTouch Ultra, LifeScan) were monitored before STZ injection and then daily afterward. Mice with glucose level above 300 mg/dl for at least 3 days were considered hyperglycemic and were injected with PV-152 as described above.

Brain slices preparation. Acute brain slices were prepared from control and STZ-treated hyperglycemic mice. After anesthesia with isoflurane, the brain was removed and immersed in ice-cold oxygenated artificial cerebrospinal fluid (aCSF) containing the following (in mM): 124 NaCl, 26 NaHCO3, 1.4 NaH2PO4, 11 glucose, 3 KCl, 1.3 MgCl2, and 1.5 CaCl2, pH 7.3–7.4. Transverse hypothalamic slices containing the PVN (300 μm) were made using a vibratome. The slices were stored in a holding chamber at 34–36°C and then transferred to a recording chamber mounted on a fixed stage under an upright microscope (Nikon FN1).

Whole cell patch-clamp recordings. Whole cell patch-clamp recordings were performed at 34–37°C on kidney-related neurons in the PVN identified under a ×40 water-immersion objective (NA = 0.8). Epifluorescence was used to identify EGFP-containing neurons and infrared illumination and differential interference contrast optics (IR-DIC) to target specific cells. For whole cell patch-clamp recordings, electrodes (3–7 MΩ) were filled with a solution containing the following (in mM): 130 KCl, 14 NaHCO3, 1.4 NaH2PO4, 11 glucose, 3 KCl, 1.3 MgCl2, and 1.5 CaCl2, pH 7.3–7.4. Transverse hypothalamic slices containing the PVN (300 μm) were made using a vibratome. The slices were stored in a holding chamber at 34–36°C and then transferred to a recording chamber mounted on a fixed stage under an upright microscope (Nikon FN1).

RESULTS

Within 3 days STZ injection resulted in hyperglycemia. The average glucose level of STZ-treated mice was 498 ± 21 mg/dl (n = 31), whereas that of controls was 172 ± 6 mg/dl. PV-152 was used to identify presympathetic kidney-related PVN neurons in control and in a mouse model of type 1 diabetes. Our recordings were conducted at 88–98 h postinoculation, and the observed EGFP labeling indicating kidney-related PVN neurons was consistent with previously published data (Cano et al. 2004).

Membrane properties of kidney-related PVN neurons in control and type 1 diabetic mice. Because very little is known about the membrane properties of kidney-related PVN neurons, first we determined the basic electrophysiological characteristics of kidney-related PVN neurons in control and STZ-treated type 1 diabetic mice. Recordings were conducted from kidney-related PVN neurons. Presympathetic kidney-related PVN neurons were identified on the basis of their green fluorescence (Fig. 1). In some cases we also tested for the presence of low-threshold spikes (LTS) as a characteristic of preautonomic PVN neurons, and we were able to observe LTS in kidney-related PVN neurons that further confirmed their preautonomic nature (Fig. 1E) (Luther et al. 2002; Luther and Tasker 2000; Stern 2001). Resting membrane potentials, input resistance, and action potential frequency were determined in kidney-related PVN neurons of control and STZ-treated hyperglycemic mice.

In control mice, one of the nine recorded kidney-related PVN neurons was considered as an outlier due to its much higher firing rate and was excluded from the analyses. The resting membrane potential of kidney-related PVN neurons in control mice was −44.6 ± 2.5 mV (range −36 to −53.4 mV, n = 8). The membrane potential of kidney-related PVN neurons in STZ-treated mice was −47.2 ± 1.8 mV (range −40.7 to −56.0 mV, n = 11, P > 0.05), indicating no significant difference in resting membrane potentials between control and hyperglycemic mice (Fig. 2C).

The input resistance is a reflection of all ionic current passing through an entire membrane surface, with the exception of capacity current. A series of current steps applied to PRV-labeled PVN neurons in current-clamp mode allowed us to identify the input resistance. The current-clamp recordings were obtained from kidney-related PVN neurons of control and type 1 diabetic mice. The input resistance of kidney-related PVN neurons in control mice was 0.98 ± 0.16 GΩ (n = 8), whereas the input resistance of kidney-related PVN neurons in STZ-treated mice was 1.05 ± 0.11 GΩ (n = 11, P > 0.05; Fig. 2D). These data indicate no significant difference in input resistance of kidney-related PVN neurons between control and hyperglycemic mice.

The firing rate of kidney-related PVN neurons of control mice was 3.2 ± 1.2 Hz (range 0–7.3 Hz, n = 8) after injection of 20-pA depolarizing current. On the other hand, in STZ-treated type 1 diabetic mice, the rate of action potential firing

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was 8.0 ± 1.7 Hz after 20-pA depolarizing current injection (range 0–12.7 Hz, n = 11, P < 0.05; Fig. 2, A and B). This finding indicates increased excitability of presympathetic kidney-related PVN neurons during type 1 diabetes.

**Decreased phasic inhibition of kidney-related PVN neurons in type 1 diabetic mice.** Previous observations indicated synaptic plasticity of presympathetic PVN neurons in rats with heart disease (Han et al. 2010). In this study, we have assessed the inhibitory control of kidney-related PVN neurons in control and type 1 diabetic conditions. Recordings of mIPSCs were conducted at −10 mV in aCSF containing 1 μM TTX to block action potential-dependent neurotransmitter release. The average frequency of mIPSCs of kidney-related presympathetic PVN neurons in slices from control mice was 0.95 ± 0.21 Hz (range 0.16–2.49 Hz, n = 12). In contrast, the frequency of mIPSCs in kidney-related PVN neurons of STZ-treated hyperglycemic animals was 0.33 ± 0.08 Hz (range 0.11–1.08 Hz, n = 12, P < 0.05; Fig. 3, A–C). Our data demonstrate significantly lower mIPSC frequency in STZ-treated type 1 diabetic mice compared with controls.
The amplitude of mIPSCs in kidney-related presympathetic PVN neurons of control mice was 18.50 ± 1.98 pA (range 10.11–32.69 pA, n = 12) and 10.00 ± 0.54 pA (range 6.05–13.85 pA, n = 12) in STZ-treated type 1 diabetic mice. The mIPSC amplitude was significantly smaller in STZ-treated mice compared with controls (P < 0.05; Fig. 3D).

The charge transfer \( Q \) measured as area under the mIPSCs was 0.15 ± 0.02 pC (range 0.05–0.35 pC, n = 12) in kidney-related PVN neurons of control mice. In type 1 diabetic mice the charge transfer was 0.08 ± 0.01 pC (range 0.03–0.18 pC, n = 12), indicating significantly smaller \( Q \) values in kidney-related PVN neurons of STZ-treated mice compared with controls (P < 0.05; Fig. 3E). We also calculated the mean current as \( Q \times \) frequency of mIPSCs. In control mice the mean current was 0.15 ± 0.04 pA (range 0.02–0.46 pA, n = 12), whereas that in type 1 diabetic animals was 0.03 ± 0.006 pA (range 0.004–0.062 pA, n = 12). This observation demonstrates significantly less mean miniature inhibitory current in type 1 diabetic mice compared with controls (P < 0.05; Fig. 3F).

The decay time was fitted well with a single exponential in 7 of 12 neurons in both control and STZ-treated mice, and two exponentials were better fitted in the remaining cells. The weighted decay time constant was 17.7 ± 2.0 ms in control and 19.6 ± 1.8 ms in STZ-treated mice. These data indicate no significant difference in decay time constant between groups (P > 0.05; Fig. 3G).

Reduced GABAergic tonic inhibitory control of kidney-related PVN neurons in type 1 diabetic mice. Tonic inhibitory current mediated by GABA\(_A\) receptors has been previously identified in PVN neurons (Park et al. 2006, 2007), supporting the idea that tonic inhibition plays a significant role in the maintenance of neuronal activity. Because autonomic impairment includes neuronal hyperactivity, we reasoned that tonic inhibitory control of kidney-related PVN neurons could be altered in type 1 diabetes.

In this study, we assessed the GABA\(_A\) receptors-mediated tonic inhibitory current at −10 mV holding potential. The bath solution contained kynurenic acid (1 mM) to block glutamatergic currents, and the tonic GABA\(_A\) current was measured as a bicuculline-dependent inward shift of the holding current (Gao and Smith 2010; Park et al. 2007). Application of bicuculline (30 \( \mu \)M) revealed a tonic inhibitory current with an average amplitude of 23.9 ± 4.0 pA (range 8.7–57.1 pA, n = 15) in kidney-related PVN neurons of control mice (Fig. 4A and C). Next, we assessed the potential effect of type 1 diabetes on tonic GABAergic inhibition of kidney-related presympathetic PVN neurons. In STZ-treated mice, administration of the same concentration of bicuculline produced a significantly smaller shift in holding current with an average amplitude of 8.8 ± 1.4 pA (range 4.1–16.3 pA, n = 8, P < 0.05; Fig. 4B and C). This set of data further indicates decreased inhibition of kidney-related PVN neurons in type 1 diabetes.
Increased mEPSC frequency in kidney-related PVN neurons of type 1 diabetic mice. Alteration of mEPSCs can also contribute to the increased excitability of presympathetic PVN neurons; therefore we investigated the excitatory control of kidney-related PVN neurons in control and type 1 diabetic conditions. The average frequency of mEPSCs in kidney-related PVN neurons in controls was $1.13 \pm 0.23$ Hz (range 0.35–3.23 Hz, $n = 13$; Fig. 5). In contrast, the frequency of mEPSCs in kidney-related PVN neurons of STZ-treated hyperglycemic animals was $2.73 \pm 0.64$ Hz (range 0.73–8.19 Hz, $n = 12$). Our data demonstrated significantly higher mEPSC frequency in type 1 diabetic mice compared with controls.

The amplitude of mEPSCs in kidney-related presympathetic PVN neurons of control mice was $13.70 \pm 0.83$ pA (range 8.52–19.21 pA, $n = 13$), and that of STZ-treated type 1 diabetic mice was $9.57 \pm 0.44$ pA (range 7.02–12.39 pA, $n = 12$). The mean current of EPSCs in controls was $0.036 \pm 0.007$ pA (range 0.01–0.1 pA, $n = 13$), whereas that in type 1 diabetic animals was $0.067 \pm 0.018$ pA (range 0.008–0.202 pA, $n = 12$), indicating an increasing trend, but the difference did not reach significance. Similar to mIPSCs, we did not reveal a difference in decay time constant of mEPSCs between control and STZ-treated mice ($3.1 \pm 0.2$ vs. $2.9 \pm 0.3$ ms, $P > 0.05$).

DISCUSSION

In the present study, we demonstrated altered regulation of kidney-related PVN neurons in a model of type 1 diabetes. The following novel findings emerged: 1) mIPSC frequency was reduced in kidney-related PVN neurons of STZ-treated type 1 diabetic mice; 2) the amplitude, charge transfer, and mean synaptic current of mIPSCs were decreased in kidney-related presympathetic PVN neurons of STZ-treated mice; 3) tonic inhibitory currents were attenuated in kidney-related PVN neurons of STZ-treated mice; and 4) increased frequency of
mEPSCs and increased excitability of kidney-related PVN neurons were observed in type 1 diabetic mice. The demonstration of decreased inhibition and increased excitability of kidney-related presympathetic PVN neurons suggests altered central autonomic circuitry, possibly contributing to elevated sympathetic nerve activity.

Technical considerations. In this study, PRV-152 was used to identify kidney-related presympathetic PVN neurons. PRV-152 has been shown to spread strictly retrogradely, with little or no ability to spread anterogradely or perform axo-axonal labeling (Pickard et al. 2002; Smith et al. 2000). Thus the EGFP expression, which can be observed under epifluorescence illumination, indicates kidney-related PVN neurons. Because the innervation of kidney originates from the sympathetic nervous system, we can assume that the EGFP labeling indicates kidney-related presympathetic neurons. Patch-clamp recordings were conducted between 88 and 98 h after inoculation of the left kidney. This time point has been shown sufficient to label neurons in the PVN (Cano et al. 2004). The possible harmful effects of PRV on neuronal survival and viability have been addressed in numerous articles (Cano et al. 2004; Card et al. 1993; McCarthy et al. 2009). Previous reports also showed no changes in electrical properties of PRV-infected neurons, indicating that the virus does not have adverse effects on electrical properties at this time point (Derbe­nev et al. 2010; Gao et al. 2012; Glatzer et al. 2003; Smith et al. 2000). Although it remains possible that PRV-152 might alter some of the labeled neurons in a later time point, our recordings were carefully designed and monitored. Nevertheless, this PRV approach provides the opportunity to identify kidney-related presympathetic PVN neurons and study their cellular properties and the consequences of diabetes.

In our experiments, mice were injected with streptozotocin to destroy pancreatic beta cells and thus induce type 1 diabetes (Like and Rossini 1976; Schein and Loftus 1968). This is a well-described, straightforward model of type 1 diabetes producing insulin deficiency and hyperglycemia. On the other hand, we have to note that STZ administration can affect renal cells and hepatic cells as well as neurons (Pabbidi et al. 2008); however, the direct effect of STZ-treatment on the PVN is negligible due to that the serum half-life of streptozotocin is ~15 min (Like and Rossini 1976; Schein and Loftus 1968). Therefore, it is highly unlikely that STZ will have direct effects on the patch-clamp recordings.

We also have to note that based on the current experimental settings, we cannot clearly determine if the observed alteration of neuronal activity is a consequence of circulating glucose or deficiency in insulin. Elevated glucose can acutely alter cellu-
lar function (Balfour et al. 2006; Ferreira et al. 2001), and chronic hyperglycemia also alters synaptic balance. On the other hand, insulin was reported to impact neuronal function in preautonomic brain stem neurons (Blake and Smith 2012). Our previous data revealed alteration of preautonomic PVN neurons during type 1 diabetes, and in vivo insulin replacement normalized neuronal function (Gao et al. 2012); however, insulin replacement also normalized glucose levels, and thus we cannot differentiate the separate effect of insulin deficiency and/or hyperglycemia. Nevertheless, our data demonstrate that in the type 1 diabetic condition, the synaptic regulation of kidney-related presympathetic PVN neurons is altered; however, future studies are required to determine the separate effect of glucose and insulin.

**Decreased inhibitory regulation of kidney-related PVN neurons in type 1 diabetes.** Autonomic dysfunction during pathophysiological conditions such as diabetes mellitus is a well-known phenomenon (Carnethon et al. 2003a, 2003b; Holl et al. 1999; Perin et al. 2001). Increased sympathetic outflow is associated with diabetes; however, the exact mechanisms driving these changes are not fully understood. Both hyperglycemia and hypoglycemia have been shown to alter cellular functions in autonomic centers of the brain (Balfour et al. 2006; Balfour and Trapp 2007; Ferreira et al. 2001; Zsombok et al. 2011), including the PVN (Gao et al. 2012). In this study, we observed reduced inhibition of kidney-related PVN neurons following 7–11 days of hyperglycemia due to STZ-treatment, even when identical normalized glucose concentrations were used in the bath solution. Our study, by using PRV-152, allowed the identification of a specific neuronal subpopulation, the kidney-related presympathetic PVN neurons, and the electrophysiological studies provided evidence that the presympathetic circuitry at least at the level of the PVN is altered during experimental type 1 diabetes. The change in circuitry may be due to increased glucose levels or lack of insulin; however, we cannot elucidate which one might count for the effect, as mentioned above. Functional plasticity of specific receptors has been previously observed in both the PVN and brain stem during type 1 diabetes (Gao et al. 2012; Zsombok et al. 2011); however, to the best of our knowledge, this is the first report indicating altered inhibitory control of kidney-related presympathetic PVN neurons in a rodent model of type 1 diabetes.

**Increased sympathetic activity is linked with many pathophysiological conditions, including heart failure, hypertension, and diabetes mellitus (Anderson et al. 1989; Li and Patel 2003; Mancia et al. 2007; Spallone et al. 1994).** Clinical studies have demonstrated that during the early course of type 1 diabetes, blood pressure is elevated compared with that of healthy subjects (Holl et al. 1999); however, the observations from animal studies are controversial (Bunag et al. 1982; Hayashi et al. 1983; Hicks et al. 1998; Katovich et al. 1995). Gradual increase of blood pressure along with hyperglycemia has also been shown in STZ-treated C57bl/sv129 mice (Wichi et al. 2007), and systolic blood pressure tends to be higher in STZ-treated mice compared with nontreated mice; however, the difference was significant only after a prolonged period (Nadarajah et al. 2012). Our data demonstrate altered control of presympathetic PVN neurons in the type 1 diabetic condition, which could play a role in the development of elevated sympathetic outflow. Sympathetic overactivity, for example, in heart failure, has been associated with reduced inhibition of rostral ventrolateral medulla (RVLM)-projecting presympathetic PVN neurons due to decrease of GABA release (Han et al. 2010). Hypertensive rats also exhibited higher basal firing rate and smaller GABA$_A$-mediated current in presympathetic PVN neurons compared with normotensive rats (Li and Pan 2006). Furthermore, the GABA$_A$ blocker-dependent increase of firing rate in PVN neurons was also blunted in hypertensive rats (Li and Pan 2006).

In STZ-treated diabetic rats, inhibition of GABAergic neurotransmission in the PVN produced a smaller renal sympathetic nerve discharge than in control animals (Reynolds et al. 1996), indicating reduced inhibitory mechanisms in the PVN during type 1 diabetes. PVN receives inputs from the brain stem and a variety of forebrain areas. It is generally accepted that presympathetic PVN neurons integrate information and regulate sympathetic outflow through direct projections to the spinal cord and RVLM, and they may also regulate sympathetic outflow indirectly via the nucleus tractus solitarii (NTS) or parabrachial nucleus (Dampney 1994; Swanson and Sawchenko 1983). Numerous neurotransmitters and neuromodulators influence the level of SNA; however, evidence has indicated that GABA plays a tonic inhibitory role in the PVN (Martin and Haywood 1993; Martin et al. 1991). It has been
suggested that increased renal SNA (RSNA) is due to increased activity of PVN sympathoexcitatory neurons as a consequence of reduced GABAergic inhibition, but increased excitation by NMDA or AT1 receptors may also contribute (LaGrange et al. 2003; Li and Patel 2003; Zucker et al. 2001). The source and location of GABAergic neurons is still debated; however, GABAergic neurons have been described in surrounding hypothalamic areas, including the lateral hypothalamic area, anterior hypothalamic area, medial preoptic area, dorsomedial hypothalamic nucleus, and suprachiasmatic nucleus (Boudaba et al. 1996). Previous in vivo data demonstrated that inhibiting GABA_A receptors by administration of bicuculline increases the level of RSNA (Zhang and Patel 1998); therefore, on the basis of our data, we can speculate that decreased GABAergic regulation of kidney-related presympathetic PVN neurons may lead to an increase of RSNA. Our data showing reduced phasic inhibitory currents suggest less GABA release to kidney-related PVN neurons. The reduced phasic current together with decreased tonic inhibition contributes to the increased excitability of kidney-related PVN neurons that could upregulate the activity of presympathetic RVLM and/or IML neurons and thus contribute to the elevated renal sympathetic outflow.

Our data demonstrating decreased mIPSC frequency, amplitude, and mean inhibitory current suggest reduction in GABA release during type 1 diabetes. Furthermore, the greater firing rate in kidney-related PVN neurons of STZ-treated type 1 diabetic mice after depolarizing current injection indicates increased excitability of the neurons. This could be due to reduced inhibitory control of PVN neurons during type 1 diabetic conditions as suggested by Reynolds et al. (1996). On the other hand, our data did not provide evidence for alteration of input resistance; however, a decrease in inhibition and an increase in excitation might counterbalance the changes in input resistance. This is supported by our findings demonstrating increased frequency of mEPSCs in STZ-treated mice. Presympathetic PVN neurons receive excitatory, glutamatergic inputs from the brain stem including the NTS (Affleck et al. 2012) and from forebrain areas including the surrounding hypothalamic nuclei (Boudaba et al. 1997; Ulrich-Lai et al. 2011). Within the hypothalamus the dorsomedial hypothalamus and the perifornical region have been identified electrophysiologically as major excitatory sites projecting to the PVN (Boudaba et al. 1997). Immunostaining studies revealed glutamatergic inputs to the posterior PVN, which contains large population of preautonomic neurons, from the ventromedial hypothalamic nucleus, posterior hypothalamic nucleus, medial amygdala, the dorsomedial hypothalamic nucleus, and lateral hypothalamic area (Ulrich-Lai et al. 2011). The higher frequency of mEPSC in type 1 diabetic mice suggests that the identified kidney-related PVN neurons may receive more excitatory regulation that could also contribute to the increased sympathetic outflow. On the other hand, no change in overall phasic current in the diabetic condition could indicate minor contribution of excitatory regulation to increased sympathetic outflow. Nevertheless, the identification of excitatory mechanisms will be the subject of future investigations.

Similar to our findings, there was no change in the input resistance and resting membrane potential in neurosecretory PVN neurons even when a shift of inhibitory-excitatory synaptic balance (Potapenko et al. 2011) was observed in presympathetic PVN neurons during heart failure (Han et al. 2010; Stern et al. 2012). Furthermore, during myocardial infarction, the spontaneous firing activity of presympathetic PVN neurons increased and both the spontaneous and miniature IPSC frequencies were reduced (Han et al. 2010). These changes were observed in the RVLM-projecting subpopulation of presympathetic PVN neurons, whereas the authors did not observe changes in the spinally projecting presympathetic PVN population (Han et al. 2010). The above-mentioned findings, including our current data, support the hypothesis that the inhibition of presympathetic PVN neurons is suppressed in pathophysiological conditions involving sympathetic overactivity.

On the other hand, no change in spontaneous and miniature IPSC frequency in dorsal horn neurons during diabetes has also been reported (Wang et al. 2007); however, the authors have demonstrated reduced presynaptic GABA_B receptor function in STZ-treated rats (Wang et al. 2007). The difference between our data and theirs could originate from the difference between spinal and central neurons, or it could be that our recordings were conducted in an early stage of diabetes (~7–11 days), whereas Wang and coworkers conducted their experiments 4 wk after STZ treatment (Wang et al. 2007).

Taken together, our data support the hypothesis that pathophysiological conditions associated with elevated sympathetic activity such as diabetes reduce the inhibitory control of presympathetic PVN neurons and increase excitability. Therefore, we can speculate that the decreased inhibition of presympathetic PVN neurons contributes to the development of sympathetic overactivity.

**Reduced GABAergic tonic inhibitory control of kidney-related PVN neurons during type 1 diabetes.** Inhibitory currents mediated by GABA_A receptors can be divided into phasic and tonic currents. Phasic currents occur when a high concentration of neurotransmitter is released from presynaptic terminal and bind with GABA receptors located in the postsynaptic cell. On the other hand, extracellular GABA, diffusing away from the synaptic cleft or originating from nonsynaptic GABA-release by glia can also activate extrasynaptic or perisynaptic GABA receptors (Nusser et al. 1998; Park et al. 2007). This low concentration of extracellular GABA continuously binds with extrasynaptic receptors, leading to the generation of tonic inhibitory currents (Gao and Smith 2010; Nusser et al. 1998; Park et al. 2007). Therefore, in addition to the conventional phasic neurotransmission, persistent tonic regulation of neurons plays a significant role in determining the activity of the neurons (Nusser and Mody 2002; Nusser et al. 1998; Park et al. 2006, 2007).

Tonic GABA_A-receptor-mediated currents were first found in cerebellar granule cells (Brickley et al. 1996) and then in many other brain areas including autonomic areas, such as the presympathetic neuronal population of the PVN (Park et al. 2007, 2009). We have demonstrated the presence of tonic GABA_A receptor-mediated inhibitory current in kidney-related presympathetic PVN neurons. The magnitude of this inhibitory current was larger in kidney-related presympathetic PVN neurons than in presympathetic RVLM-projecting PVN neurons (~24 vs. ~10 pA; Park et al. 2007). We can speculate that this difference could be due to a greater level of tonic inhibition of kidney-related PVN neurons compared with the overall RVLM-projecting presympathetic PVN population or to different concentrations of bicuculline (30 vs. 20 μM) or a species difference (mouse vs. rat) (Park et al. 2007). Regardless of the magnitude of the tonic inhibitory current, our data also dem-
onstrate the existence of GABA<sub>A</sub>-dependent persistent tonic inhibition of presympathetic PVN neurons, and thereby confirm and even further extend the previous findings.

As important additional information, our work revealed that in type 1 diabetic conditions, the tonic GABAergic inhibitory current in kidney-related presympathetic PVN neurons was significantly smaller than in control mice. Because sympathetic outflow from the PVN is restrained by a GABAergic inhibitory tone, we can assume that decreased tonic inhibition during diabetic conditions could lead to hyperactivity of PVN neurons and could be associated with increased renal sympathetic outflow; however, future experiments are required to prove this possibility. This also could be supported by previous findings demonstrating blunted renal sympathetic responses in diabetic rats following bicuculline injection into the PVN (Reynolds et al. 1996).

In summary, the current study extends our understanding of synaptic regulation of kidney-related presympathetic PVN neurons. Furthermore, we have demonstrated increased excitability and reduced inhibition of kidney-related PVN neurons in type 1 diabetic conditions. These data have valuable implication given that they provide a possible mechanism associated with increased sympathetic outflow during pathophysiological conditions such as diabetes mellitus.

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